CORRESPONDENCE

Alveolar levels of immuno-inflammatory mediators in diffuse alveolar hemorrhage after allogeneic transplant

Lisa K. Vande Vusse[1,2] · Mark M. Wurfel[1] · David M. Madtes[1,2] · H. Gary Schoch[2] · Susanna Harju-Baker [1] ·
Joshua A. Hill [2,3] · Keith R. Jerome[2,3] · Michael Boeckh[2,3] · Timothy R. Watkins[1]

Idiopathic pneumonia syndrome (IPS) is a severe and morbid complication of hematopoietic cell transplantation (HCT) that largely lacks effective treatments. The syndrome is defined as abnormal pulmonary physiology with multi-lobar opacities on chest imaging that are not attributable to lower respiratory tract infection (LRTI) or other organ failures [1]. Many pathologies meet IPS criteria, including organizing pneumonia, interstitial pneumonitis, and diffuse alveolar damage, among others. Biologic heterogeneity within this broadly defined syndrome may diminish the ability to detect the effect of treatments. An improved understanding of IPS pathobiology may catalyze the development of effective preventive and therapeutic strategies [2].

Several pro-inflammatory cytokines/chemokines are elevated in IPS [3, 4]. These biomarkers may be useful to distinguish IPS subphenotypes with unique biologic features. As a preliminary examination of this concept, we tested the hypothesis that immuno-inflammatory mediators measured in the blood and alveolar compartments of people with IPS differ according to the presence or absence of diffuse alveolar hemorrhage (DAH). DAH is a variably present manifestation of IPS that is proposed to have distinct biology [5]. We retrospectively studied 17 adults who underwent clinical bronchoscopy for diffusely abnormal chest imaging within 120 days of allogeneic HCT at Fred Hutchinson Cancer Research Center (FHCRC) between

2008 and 2010. None received specific tumor necrosis factor (TNF)-α inhibitors prior to bronchoscopy. Data and samples were banked prospectively with subjects' consent under FHCRC protocol 999.209 and were approved for use in this research (FHCRC protocols 808 and 1829/substudy 050).

We reviewed pertinent progress notes, microbiology reports, pathology reports, bronchoscopy reports, and radiographic images to identify IPS cases according to the American Thoracic Society definition [1]. We defined abnormal respiratory physiology as oxygen saturation ≤92% by pulse oximetry or arterial blood gas while breathing ambient air or supplemental oxygen. We defined LRTI as bronchoalveolar lavage fluid (BALF) growing any amount of Gram-negative rods, *Mycoplasma* spp., *Chlamydophila* spp., fungi/mold, *Legionella*, *Nocardia*, *Mycobacteria*, or >10^4 colony-forming units per mL of a single pathogenic Gram-positive coccus. Cytomegalovirus (CMV) infection required both culture and PCR positivity. We also classified as LRTI: any positive direct fluorescence antibody stain or >40 PCR copies of respiratory viruses (such as adenovirus, coronavirus, parainfluenza, influenza A/B, respiratory syncytial virus, human metapneumovirus, herpes simplex, and varicella zoster); positive stain for *Pneumocystis jirovecii*; and BALF or serum galactomannan >0.5 ng/L. DAH required documentation of increasingly bloody return from serial bronchoalveolar lavages, or report of "bloody" BALF followed by treatment with systemic corticosteroids for clinically diagnosed DAH.

According to local protocols, blood samples were drawn at weekly intervals to survey for CMV and processed and cryopreserved immediately using local standard operating procedures. When available, we selected blood samples collected prior to bronchoscopy or within 2 days of bronchoscopy to best reflect biologic activity at the time of bronchoscopy. BALF samples were obtained using standard clinical techniques and cryopresevered without processing. We thawed samples on an ice batch and centrifuged them at

✉ Lisa K. Vande Vusse
lkvandev@u.washington.edu

1   Division of Pulmonary and Critical Care, University of Washington, Seattle, WA, USA

2   Clinical Research Division, Fred Hutchinson Cancer Center Research Center, Seattle, WA, USA

3   Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

1000×g for 10 min to remove debris. We then batch-analyzed in duplicate a broad array of immuno-inflammatory cytokines, chemokines, and growth factors using commercially available multiplexed immunoassay kits per manufacturer guidelines (Meso Scale Diagnostics™, Rockville, MD). We generated standard curves using serial dilutions of standard sample matrices of known concentration and determined analyte concentrations using Meso Scale Discovery Workbench version 4.0 (copyright 2007).

Individual analytes with a concentration 0.5 SD or less above the mean signal for the blank control were replaced with a value of 0 pg/mL (analyte $n = 149$). Analytes with a signal between 0.5 and 2.5 SD above the mean signal for the blank were left unchanged. We performed two-way analysis of variance, and based on intra-class correlation coefficients <0.7, interleukin (IL)-2, IL-12 p70, IL-4, and TNF-β were dropped for both BALF and plasma. In addition, IL-5, IL-10, IL-13, Macrophage-Derived Chemokine (MDC), and Thymus and activation-regulated chemokine (TARC) were dropped for BALF and TNF-α was dropped for plasma. We summarized concentrations of replicate samples as means and used two-sample Wilcoxon rank-sum tests for statistical comparisons. The statistical analysis was planned and performed by research team members with relevant expertise (Vande Vusse, Wurfel; computer code requests can be addressed to the corresponding author). We used StataIC 12.0 software (Stata-Corp LP, College Station, TX) for data analysis and defined statistical significance as a two-sided, $P < 0.05$.

Eleven subjects met criteria for IPS—six with DAH and five without. Five subjects had evidence of LRTI. We excluded one subject with congestive heart failure from all analyses. Several conditioning regimens were used at the clinical providers' discretion. Mortality was high in all groups (Table 1).

Cytokine and chemokine levels were highly variable (Table 2). BALF concentrations of IL-15, MIP-1α (CCL3), MIP-1β (CCL4), and MCP-1 were higher in subjects with IPS and DAH than those with non-DAH IPS. No blood biomarker differed between these groups (data not shown). The biomarkers elevated in BALF from subjects with DAH mediate the recruitment and activation of innate and adaptive immune effector cells [6–10]. Our findings are consistent with higher levels of immune system activity in the alveolar compartment of people with IPS and DAH than those with non-DAH IPS. DAH may be the clinical manifestation of unique lung-localized pathobiology that would not be recognized measuring blood biomarkers alone.

Given the number of comparisons made in our broad examination of biomarkers, some of our statistically significant findings may be false positives. Both preclinical and clinical studies have contributed importantly to the current

Table 1 Subject, stem cell transplant, and sampling characteristics

Characteristic[a]	LRTI (n = 5)	IPS with DAH (n = 6)	IPS without DAH (n = 5)
Age (years), mean (SD)	45 (15)	37 (7)	49 (9)
Male	4 (80)	4 (67)	3 (60)
White race	4 (80)	2 (33)	4 (80)
Acute leukemia	3 (60)	3 (50)	4 (80)
Myeloablative conditioning	3 (60)	4 (67)	4 (80)
Any total body irradiation	3 (60)	2 (33)	3 (60)
Stem cell source			
Bone marrow	2 (40)	3 (50)	1 (20)
Peripheral blood	1 (20)	2 (33)	3 (60)
Cord blood	2 (40)	1 (17)	1 (20)
HLA match and donor relation			
Matched, related	0	2 (33)	1 (20)
Matched, unrelated	4 (80)	2 (33)	2 (40)
Mismatched	1 (20)	2 (33)	2 (40)
Engrafted before bronch	2 (40)	2 (33)	3 (60)
GVHD before bronch	3 (60)	2 (33)	2 (40)
Days HCT to bronch, mdn (IQR)	20 (19–36)	14 (9–19)	34 (24–59)
Days bronch to blood sample, mdn (IQR)	1 (1–1)	5 (2–7)	1 (1–1)
Died ≤90 days from bronch	4 (80)	3 (50)	2 (40)

LRTI lower respiratory tract infection, *IPS* idiopathic pneumonia syndrome, *DAH* diffuse alveolar hemorrhage, *HLA* human leukocyte antigen, *GVHD* acute graft-versus-host disease, *bronch* bronchoscopy

[a] Values are number of cases with percents in parentheses, unless otherwise noted

understanding of IPS. Reassuringly, our results are similar to those found in experimental models and clinical settings. Panoskaltsis-Mortari et al. examined lung protein extracts, serum, and BALF from a murine IPS model exposed to cyclophosphamide, total body irradiation, allogeneic HCT, and natural killer (NK)-cell depleted spleen cell infusion (a T-cell source) [11]. This study demonstrated time-varying elevations in MIP-1α and MCP-1 messenger RNA expression and protein production in all compartments relative to healthy controls. In another preclinical study, MIP-1α, MIP-1β, and MCP-1 were expressed early after irradiated mice received allogeneic bone marrow transplants [12]. Experimental disruption of either MCP-1 or its receptor lowered BALF monocyte, macrophage, and in the latter case, T-cell counts and lessened histopathologic lung injury. Clinical studies demonstrate that MCP-1 levels are elevated in BALF collected from people with IPS relative to healthy controls and individuals with chronic noninfectious lung

Table 2 Concentrations of immuno-inflammatory markers in bronchoalveolar lavage fluid

	IPS with DAH (n = 6)		IPS without DAH (n = 5)		
	Median (pg/mL)	IQR	Median (pg/mL)	IQR	P
IFN-γ	12.8	24.3	0.5	4.3	0.07
IL-12 p 40	5.6	28.1	2.5	2.0	0.36
IL-17A	3.8	10.9	0.0	0.0	0.07
IL-13	10.5	8.4	7.8	2.7	0.58
TNF-α	4.1	31.4	1.6	0.5	0.27
IL-1β	3.2	5.2	1.8	1.4	0.27
IL-6	2933.0	3335.5	77.7	2169.8	0.07
IL-8	1785.1	19,426.0	249.2	167.3	0.10
MIP-1α	96.7	222.2	21.1	17.7	0.02
MCP-1	6402.3	4850.1	612.7	2251.2	0.01
GM-CSF	49.3	137.7	5.0	25.3	0.10
Eotaxin	88.0	185.8	52.8	69.7	0.36
Eotaxin-3	9.7	11.2	1.8	5.3	0.10
IL-15	14.6	18.7	5.9	5.3	0.03
IL-16	188.6	183.3	181.9	104.6	0.86
IL-1α	5.9	8.2	2.2	2.6	0.36
IL-7	1.5	5.3	1.9	6.4	0.58
IP-10	305.5	529.1	57.4	1645.0	0.92
MIP-1ß	213.9	373.0	15.4	6.0	0.03
VEGF	89.3	34.5	77.2	28.9	1.00
MCP-4	170.2	1607.1	25.0	232.0	0.25

disease after HCT [4, 12]. In one observational study, BALF sampled before and after TNF-α antagonist therapy demonstrated decreases in MCP-1 and other inflammatory mediators in the subset of subjects who experienced clinical improvement [4]. Our study extends these findings by suggesting that IPS subtypes may have different inflammatory profiles and therefore differing response to immunomodulatory therapies.

A recent study applied modern molecular diagnostic techniques to BALF samples from individuals previously diagnosed with IPS and found occult viruses of unknown clinical significance [13]. In a post hoc analysis, we examined the distribution of the most prevalent occult virus known to be a human pathogen, HHV-6, using a real-time quantitative fluorescent probe PCR assay as previously described [14, 15]. Eight of the 17 study subjects were HHV-6 positive. HHV-6 was similarly distributed between subjects with and without IPS and between subjects with IPS and DAH and subjects with non-DAH IPS. BALF and plasma cytokine levels did not differ between IPS cases with HHV-6 and IPS cases without HHV-6 (data not shown). Although it is possible that the current IPS definition misclassifies some infections, HHV-6 did not clearly drive the findings of our study. Future research must consider the potential role of "occult" viral infection when defining IPS subphenotypes.

This research has several potential limitations. This small study lacking at-risk controls may be underpowered to detect differences. The lack of standard methods to correct for dilution of BALF may introduce error. The impacts of therapeutic corticosteroids, the cryopreservation of unprocessed BALF, and variable timing of sample collection on our results are unknown. The onset of non-DAH IPS occurred later than IPS with DAH and more often after engraftment. In this study, DAH may be a surrogate for important biologic differences between early- and later-onset IPS. Alternatively, the differences we found may relate to time passing instead of varied biology. A confirmatory prospective validation study is needed that ideally collects and processes plasma and BALF simultaneously. Analysis of protein levels, leukocyte expression, and cellular profiles may help elucidate important biologic differences between IPS subtypes.

In conclusion, the immune system is particularly active in the DAH phenotype of IPS occurring within 120 days of allogeneic HCT. The relevance of this small study lies in its potential to influence future research. Our findings support the hypothesis that IPS includes a spectrum of pathologies that may differentially respond to risk factor modification and therapies. The heterogeneous nature of the current IPS definition may not allow research to reveal the true effects of immune modulating therapies. Future research is needed to identify biologically distinct IPS subphenotypes as progress toward precision medicine and improved outcomes.

Compliance with ethical standards

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